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Thiol reactivity as a sensor of rotation of the converter in myosin

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Abstract

Smooth muscle myosin has two reactive thiols located near the C-terminal region of its motor domain, the "converter", which rotates by ~70° upon the transition from the "nucleotide-free" state to the "pre-power stroke" state. The incorporation rates of a thiol reagent, 5-(((2-iodoacetyl)amino)ethyl)aminonaphthalene-1-sulfonic acid (IAEDANS), into these thiols were greatly altered by adding ATP or changing the myosin conformation. Comparisons of the myosin structures in the pre-power stroke state and the nucleotide-free state explained why the reactivity of both thiols is especially sensitive to a conformational change around the converter, and thus can be used as a sensor of the rotation of the converter. Modeling of the myosin structure in the pre-power stroke state, in which the most reactive thiol, "SH1", was selectively modified with IAEDANS, revealed that this label becomes an obstacle when the converter completely rotates toward its position in the pre-power stroke state, thus resulting in incomplete rotation of the converter. Therefore, we suggest that the limitation of the converter rotation by modification causes the as-yet unexplained phenomena of SH1-modified myosin, including the inhibition of 10S myosin formation and the losses in phosphorylation-dependent regulation of the basic and actin-activated Mg–ATPase activities of myosin.

Keywords: Ca^{2+}-regulation; Smooth muscle myosin; Phosphorylation; Regulatory light chains; Contraction; Mg–ATPase; Thiol modification

Contraction and relaxation of muscles are regulated by the intracellular Ca^{2+} concentration. The interaction between vertebrate skeletal actin and myosin is controlled by troponin–tropomyosin, which binds to the actin filament in a Ca^{2+}-dependent manner [1]. In contrast, the actin-myosin interaction of smooth muscle or non-muscle cells is regulated by the phosphorylation of myosin regulatory light chains (RLCs), which is mediated by Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) [2–5]. Nevertheless, chemical modifications of a reactive thiol of both myosins lead to a complete loss of Ca^{2+}-regulation of both contractile systems [6–10]. The goal of our study is to understand the reason for this peculiar similarity, even though skeletal and smooth muscles have different regulatory mechanisms.

Skeletal and smooth muscle myosins have a conserved thiol, "SH1", which is located on the 20-kDa C-terminal segment of the subfragment-1 (a single myosin head, S1) heavy chain [11–13]. In both myosins, modification of SH1 abolished the K (EDTA)–ATPase activity and increased the Ca–ATPase activity. When rabbit skeletal myosin was reacted with iodoacetamide or its derivative compounds, e.g., 5-(((2-iodoacetyl)amino)ethyl)aminonaphthalene-1-sulfonic acid (IAEDANS), SH1 was selectively modified [14]. Using this unique reactivity, it was shown that the modification of SH1 is linearly correlated with a loss in the Ca^{2+}-regulation of the actin-activated

Abbreviations: HMM, heavy meromyosin; S1, subfragment-1; MD, motor domain; MDE, motor domain plus essential light chain; RLC, regulatory light chain; ELC, essential light chain; MLCK, myosin light chain kinase; NTM, native tropomyosin; NEM, *N*-ethylmaleimide; IAEDANS, 5-(((2-iodoacetyl)amino)ethyl)aminonaphthalene-1-sulfonic acid; AEDANS, 5-(((2-acetyl)amino)ethyl)aminonaphthalene-1-sulfonic acid.

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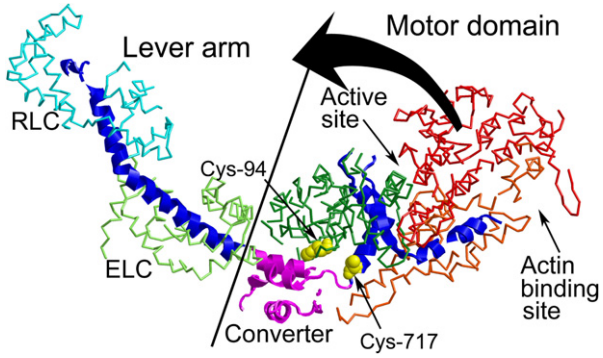

Fig. 1. The locations of the two reactive thiols in myosin S1. The N-terminal 25-kDa (residues 4–204), the upper 50-kDa (207–468), and the lower 50-kDa (469–651) segments of the myosin S1 heavy chain are colored green, red, and orange, respectively. The converter (720–791) and the two C-terminal segments except for the converter (652–719 and 792–869) are colored magenta and dark blue, respectively. ELC and RLC are colored light green and cyan, respectively. Two reactive thiols, at positions 94 and 717, are depicted by yellow balls. The crystal structure of chicken skeletal myosin S1 with no nucleotide is adapted from Ref. [41], but the residues have been labeled as though they were the homologs of the smooth muscle sequence. The curved arrow indicates the direction of the movement of the motor domain when the nucleotide-free state changes into the pre-power stroke state. Therefore, the myosin head in the pre-power stroke state bends around its converter region.

Mg–ATPase activity by troponin–tropomyosin [7]. The reactions of smooth muscle myosin with thiol reagents are more complicated. At least two thiols on its heavy chain and a thiol on its essential light chain (ELC) were reactive to IAEDANS [10] or *N*-ethylmaleimide (NEM) [9,15]. The two heavy chain thiols of chicken gizzard myosin were identified as Cys-94[1] and Cys-717 [13,17]. The former was substituted with a histidine in the rabbit skeletal sequence, while the latter was a conserved cysteine of the SH1 type. Both cysteines are spatially close to the converter, but far from other functional sites, e.g., the active site of ATP hydrolysis, the actin binding site, and the phosphorylation site of the RLC (Fig. 1). The converter, which is the C-terminal region of the motor domain, is directly connected with the lever arm, by which myosin movement delivers a mechanical force to actin filaments. A conformational change in the motor domain induced by nucleotide binding causes a ~70° rotation of the converter, resulting in a large swinging motion of the rigidly attached lever arm. Since the rates of incorporation are differently affected by adding ATP or varying the KCl concentration, one can separately determine the effects of their incorporation into SH1 and other thiols. Upon the addition of ATP, the rate of incorporation into SH1 was decreased for IAEDANS [10], while it was increased for NEM [9,15]. In spite of this difference, SH1 modifications with both reagents caused a complete loss of the regulation of the actin-activated Mg–ATPase activity of smooth muscle myosin by phosphorylation [9,10,15]. Interestingly, these reagents also inhibited the formation of the 10S conformation [9,15], stabilized the myosin filaments in the presence of ATP [9,10,15], and abolish the phosphorylation-dependent regulation of the Mg–ATPase activity of the myosin in the absence of actin [10]. We will review our previous study on thiol modification of smooth muscle myosin and will reinvestigate why the SH1 modification can functionally mimic the phosphorylation of RLCs, even though the position of SH1 is far away from the myosin neck region, where phosphorylation sites reside. Finally, we infer how the SH1 modification of skeletal myosin disables the blocking of the interaction between actin and myosin by troponin–tropomyosin.

Materials and methods

Protein preparation. Myosin and native tropomyosin (NTM) were prepared from chicken gizzard, as previously described [18]. F-actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt [19].

Sedimentation study. Centrifugation was conducted at 20 °C in a Beckman model E ultracentrifuge. Sedimentation coefficients were estimated from a plot of the logarithm of r (the distance from the center of rotation) versus time, with corrections for solvent viscosity and density. The partial specific volume of gizzard myosin was assumed to be the same as that of skeletal myosin (0.729 ml/g).

Modeling of myosins with the SH1 group modified with IAEDANS. The structure of AEDANS-cysteine was optimized using the semi-empirical molecular orbital method program MOPAC, in the Chem3D software suite (CambridgeSoft Corp., Cambridge, MA). The model of SH1-modified myosin in the nucleotide-free state was produced by replacing AEDANS-cysteine with Cys-717 from the crystal structure of chicken skeletal S1 with no nucleotide (PDB: 2MYS). Before replacement, missing hydrogen atoms were added to the S1 structure. All of the coordinates, except for those of AEDANS-cysteine were fixed, while the AEDANS-cysteine portion was energy-minimized, and then simulated annealing from 1000 K to 298 K was performed using the CNS program [20]. Independent annealing was carried out 20 times. The annealed structures were energy-minimized once more. On the other hand, the model of SH1-modified myosin in the pre-power stroke state was constructed by using AEDANS-cysteine and the crystal structure of gizzard MDE with MgADP·AlF_4^- (PDB: 1BR1). However, the SH1-containing cleft of MDE was not large enough to fuse AEDANS and Cys-717. In order to widen the cleft, the C-terminal domain, starting from Gly-709, was rotated by ~15° toward its position in the nucleotide-free state. Cys-717 was then replaced with AEDANS-cysteine, and the structure was subsequently energy-minimized and annealed as described above.

Results and discussion

The IAEDANS modification rates of the two thiols of the myosin heavy chain were altered by adding ATP or changing the myosin conformation

At physiologically low ionic strength (0.15 M KCl), thick filaments of unphosphorylated gizzard myosin are

[1] Although the amino acid sequences of a protein differ in various organisms (or in different major tissues of the same organism), well-established homologies permit interspecific translation from one species to another. Throughout this report, we use the sequence numeration appropriate for smooth muscle myosin, as extracted from chicken gizzard [16]. We note that Cys-94, Gly-118, Leu-119, Gly-709, Cys-717, Pro-722, Thr-778, and Gly-779 correspond to chicken skeletal His-96, Gly-120, Leu-121, Gly-699, Cys-707, Pro-712, Ala-769, and Gly-770, respectively, and to *Dictyostelium discoidium* Tyr-96, Gly-120, Leu-121, Gly-680, Thr-688, Pro-703, Ala-748, and Gly-749, respectively.

dissociated by ATP [21], into monomers with a sedimentation coefficient of about 10S [22,23]. The 10S monomer has two distinct structural features from the 6S monomer with an extended tail; i.e., the myosin tail is folded through interactions with the neck region, and two myosin heads bend back toward the tail [23,24]. On the other hand, myosin in 0.3 M or higher concentrations of KCl exists in the 6S monomer and remains unchanged even in the presence of ATP. In 0.2 M KCl, an intermediate concentration, the 6S monomer is converted into the 10S monomer by the addition of ATP [22].

To determine whether the modification of myosin thiols is affected by adding ATP or changing the myosin conformation from 6S to 10S, unphosphorylated gizzard myosin was reacted with IAEDANS in four concentrations (0.15 M, 0.2 M, 0.3 M, and 0.4 M) of KCl and in the presence or absence of ATP. After the reactions were terminated with 2-mercaptoethanol, the samples were digested with trypsin and analyzed by SDS–PAGE. Under the conditions used, trypsin cleaved the myosin heavy chain into 24-kDa, 50-kDa, 68-kDa, and 92-kDa fragments in order from its N-terminal end. The N-terminal 24-kDa fragment (colored green in Fig. 1) contains Cys-94, and the 68-kDa fragment includes the C-terminal 20-kDa segment of the S1 heavy chain (blue and magenta ribbons in Fig. 1) bearing Cys-717 (“SH1”). The amount of AEDANS incorporated into each fragment was determined by measuring its fluorescence intensity with a gel scanner. When myosin was modified in the absence of ATP, the AEDANS was rapidly incorporated into the 68-kDa fragment, independently of the KCl concentration tested (Fig. 2A). However, the incorporation in the presence of ATP was dependent on the KCl concentration. In 0.15 M or 0.2 M KCl, the incorporation was strongly inhibited, while in 0.3 M or 0.4 M KCl, the rapid incorporation was inhibited, but the slow incorporation occurred subsequently until 90 min of incubation. This result suggests that ATP weakly inhibits the reaction of SH1 with IAEDANS when the myosin remains in the 6S form, while the inhibition is further strengthened by the transition from the 6S to 10S form. The incorporation into the 24-kDa fragment was affected by ATP, but not by varying the KCl concentration (Fig. 2B). In the absence of ATP, a small amount of AEDANS was incorporated within 6 min (the first time for sampling), and the slow incorporation in the subsequent stage occurred. In the presence of ATP, the rapid incorporation was unaffected, but the slow incorporation was enhanced. Therefore, the effective modification of Cys-94 with IAEDANS requires the presence of ATP, but is unaffected by the 6S–10S transition. The incorporation into the ELC was unaffected by adding ATP or varying the KCl concentration (data not shown).

Seidel and his colleagues reported that ATP accelerates the incorporation of NEM into SH1 when myosin exists in the 6S form [9,15]. This result was apparently opposite to our result obtained in 10 mM $MgCl_2$, but their experiments were generally carried out in 0.1 mM EDTA. We reexamined the modifications using IAEDANS, but in the presence of 10 mM EDTA instead of $MgCl_2$. Our result was essentially the same as their reported result (Fig. 2C). Thus, we cannot agree with their conclusion that ATP has opposite effects on the reactivity of SH1 to NEM and IAEDANS when myosin remains in the 6S form. Different effects on the SH1 modification of MgATP (Fig. 2A) and Mg^{2+}-free ATP (Fig. 2C) suggest that the Mg^{2+}-binding to the active site gives a significant influence on the ATP-induced conformational change of myosin. The role of Mg^{2+} is also supported by our unpublished observation that gizzard myosin at 0.15 M KCl can assume the 10S form in the presence of MgATP, but cannot in the presence of Mg^{2+}-free ATP.

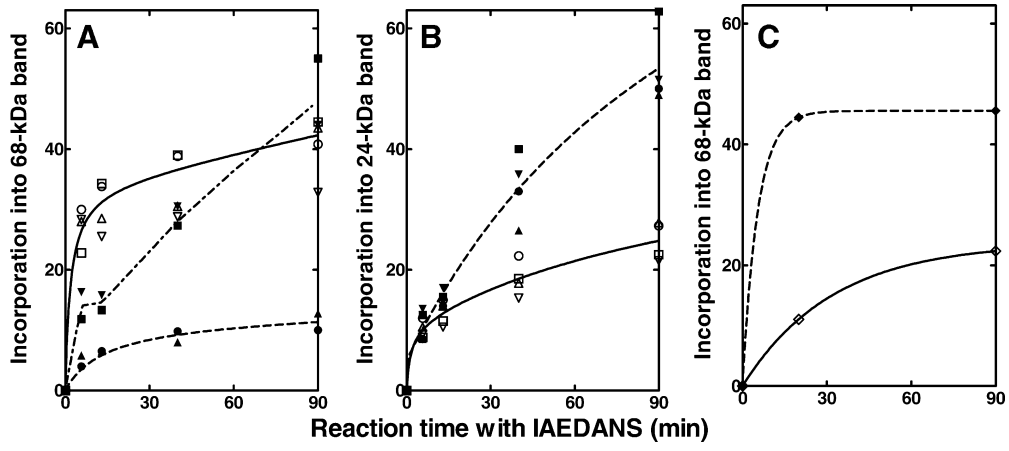

Fig. 2. Effects of MgATP and the KCl concentration on the incorporation of IAEDANS into the 68-kDa tryptic fragment (A) and the 24-kDa tryptic fragment (B), and the effect of Mg^{2+}-free ATP on the IAEDANS incorporation into the 68-kDa tryptic fragment (C). (A) and (B) are cited from Ref. [10]. Unphosphorylated gizzard myosin (2.5 mg/ml) was modified at 20 °C with 0.8 mM IAEDANS for the indicated times, in a solution containing 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5), and various KCl concentrations (0.15 M, ○, ●; 0.2 M, △, ▲; 0.3 M, □, ■; or 0.4 M, ▽, ▼), or 10 mM EDTA, 20 mM Tris–HCl (pH7.5), and 0.15 M KCl (◇, ◆). The solution also contained 1.5 mM ATP (closed symbols) or no ATP (open symbols). The reaction was stopped with 30 mM 2-mercaptoethanol. Modified myosins were digested with 1/100 (w/w) trypsin at the same temperature for 60 min, in a solution containing 0.3 M KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5), and 0.3 mM DTT.

After the modification of SH1 with IAEDANS, smooth muscle myosin was no longer regulated by the phosphorylation of RLCs

Since the reactivity of SH1 is affected by ATP and by varying the KCl concentration, we investigated the effects of the ATP and KCl concentrations on the AEDANS-induced changes of the basic Mg–ATPase activity of unphosphorylated and phosphorylated myosins (Fig. 3A). Since gizzard NTM in the ATPase assay medium contained MLCK and calmodulin, the RLCs were completely phosphorylated very early in the assay period in the presence of $CaCl_2$, but not in its absence. At the reaction time 0 with IAEDANS, the activity of the unphosphorylated native myosin was $0.0066\ s^{-1}\ head^{-1}$ and was increased to $0.02\ s^{-1}\ head^{-1}$ by the phosphorylation of RLCs (sensitivity to phosphorylation ≈3). When myosin was modified in the absence of ATP, the activities of both the phosphorylated and unphosphorylated myosins increased rapidly and reached the same maximum of about $0.06\ s^{-1}\ head^{-1}$, thus resulting in a complete loss of the response to phosphorylation. From this result, we assume that the modification gives double effects on the activity of the unphosphorylated myosin. One is the increase by a loss of the response to phosphorylation, and the other is the further threefold increase of the activity, which is independent of the phosphorylation. Only the threefold increase was observed with the phosphorylated modified myosin. When the modification was conducted in the presence of ATP and at 0.3 M KCl, both activities increased at a slow rate, and the response to phosphorylation was abolished at 90 min. When the modification was carried out in the presence of ATP and at 0.15 M KCl, the activities of both the phosphorylated and unphosphorylated myosins were practically unaffected, even after 90 min of modification. Since dependences of the activities of the modified myosins on the ATP and KCl concentrations of the modification are parallel to those of the incorporation of AEDANS into SH1, but not to those of the incorporation into other thiols, both the loss of the response to phosphorylation and the further threefold increase of the activity must be due to the modification of SH1 and not to that of any thiol other than SH1.

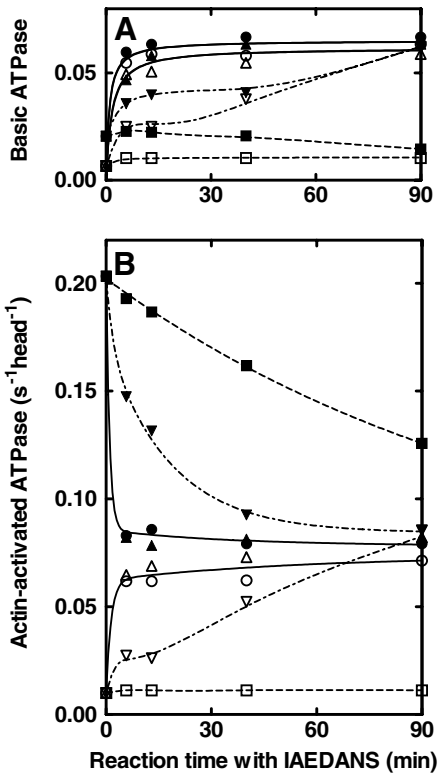

Fig. 3. Changes in the basic (A) and actin-activated (B) Mg–ATPase activities of gizzard myosin induced by reaction with IAEDANS (cited from Ref. [10]). Unphosphorylated gizzard myosin was modified with 0.8 mM IAEDANS at 20 °C for the indicated times, in a solution of 10 mM $MgCl_2$ and 20 mM Tris–HCl (pH 7.5) containing 0.15 M KCl (○, ●), 0.15 M KCl and 1.5 mM ATP (□, ■), 0.3 M KCl (△, ▲), or 0.3 M KCl and 1.5 mM ATP (▽, ▼). The activity in the absence or presence of 0.1 mg/ml rabbit skeletal actin was measured at 20 °C with 0.05 mg/ml chicken gizzard NTM, in the presence of 1 mM EGTA (open symbols) or 0.1 mM $CaCl_2$ (closed symbols), to obtain results with unphosphorylated and phosphorylated myosins, respectively. The assay medium also contains 0.1 mg/ml myosin, 0.07 M KCl, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5), 0.3 mM DTT, and 1 mM ATP.

We also investigated the effects of the ATP or KCl concentrations of the modification on AEDANS-induced changes of the actin-activated Mg–ATPase activity of phosphorylated and unphosphorylated myosins (Fig. 3B). With untreated myosin (at the reaction time 0 with IAEDANS), actin increased the activity of the phosphorylated state by 10 times to $0.2\ s^{-1}\ head^{-1}$, but did not affect the activity of the unphosphorylated state at all. Actin thus increased the sensitivity to phosphorylation by 10 times. When myosin was reacted with IAEDANS in the absence of ATP and in 0.15 M or 0.3 M KCl, the activities of phosphorylated myosin rapidly decreased, while those of unphosphorylated myosin quickly increased, both to about $0.08\ s^{-1}\ head^{-1}$. Thus, the loss of the sensitivity to phosphorylation quickly occurred. When the Mg–ATPase activities of the phosphorylated native and phosphorylated IAEDANS-treated myosins were compared, the modification increased the basic activity from $0.02\ s^{-1}\ head^{-1}$ to about $0.06\ s^{-1}\ head^{-1}$ (Fig. 3A), but decreased the actin-activated activity from $0.2\ s^{-1}\ head^{-1}$ to about $0.08\ s^{-1}\ head^{-1}$ (Fig. 3B). The net actin activation calculated by subtracting the basic activity from the actin-activated activity was thus decreased by over 90% by the modification. When myosin was incubated with IAEDANS in the presence of ATP and in 0.15 M KCl, the actin-activated activity of phosphorylated myosin was decreased very slowly, and that of the unphosphorylated myosin practically unaffected. Therefore, the sensitivity to phosphorylation was only slowly decreased. When myosin was modified in the presence of ATP and in 0.3 M KCl, the actin-activated activities of the phosphorylated and unphosphorylated myosins were changed at an intermediate rate. Since dependences of the activities of the modified

myosins on the ATP and KCl concentrations of the modification are parallel to those of the AEDANS incorporation into SH1, the modification of this thiol solely results in both the loss of response to phosphorylation and the over 90% reduction of the net actin activation of the phosphorylated myosin.

SH1-modified myosin remained in the 6S form, even in the presence of ATP

To determine whether the reaction of IAEDANS with SH1 inhibits the formation of 10S myosin, myosin was modified with IAEDANS for 30 min in the presence of 0.15 M or 0.3 M KCl with and without ATP, and the sedimentation velocity was measured in the presence of ATP and in 0.15 M or 0.2 M KCl (Table 1). In centrifugation under these conditions, unphosphorylated untreated (native) myosin sedimented as a single peak of about 10S. When the reaction with IAEDANS was carried out in the absence of ATP, and thus SH1 was highly reactive, the myosin sedimented very rapidly in 0.15 M KCl, with a sedimentation coefficient of 177S, while it sedimented at a velocity of about 6S in 0.2 M KCl. These results suggest that the SH1 modification abolishes the ability to form 10S myosin and thus stabilizes the thick filaments against the dissociating action of ATP at 0.15 M KCl. Appearance of a 10S peak, when the reaction with IAEDANS was conducted in the presence of ATP and in 0.15 M or 0.3 M KCl, where SH1 is unreactive and less reactive, respectively, provides evidence that the modification of other thiols, besides SH1, does not affect the formation of 10S myosin.

Modification rates of two heavy-chain thiols are sensitive to the position of the converter

To understand why the rates of modification of the two heavy chain thiols are changed upon the addition of ATP (that is, the conversion from the nucleotide-free state to the pre-power stroke state), we investigated the nearest neighbors of Cys-717 (SH1) and Cys-94 in the crystal structures of chicken skeletal S1 with no nucleotide (panels A and B of Fig. 4, respectively), chicken gizzard myosin motor domain plus ELC (MDE) with MgADP·AlF_4^- (panels E and F, respectively), and *Dictyostelium* myosin motor domains (MDs) with no nucleotide (panels C and D, respectively) and with MgADP·VO_4^- (panels G and H, respectively). Skeletal S1 and *Dictyostelium* MD, both with no nucleotide, represent a state before the rotation of the converter[2], while gizzard MDE with MgADP·AlF_4^-

Table 1
Sedimentation coefficients of native and treated myosins

Myosin	Modification conditions		Sedimentation coefficient at	
	[KCl] (M)	[ATP] (mM)	0.15 M KCl (S)	0.2 M KCl (S)
Native myosin	—	—	10.3	8.9
Modified myosin	0.15	0	177	5.8
	0.15	1.5	10.3	9.4
	0.3	0		6.0
	0.3	1.5		6.0 + 9.5

Unphosphorylated myosin (2.5 mg/ml) was incubated with 0.8 mM IAEDANS for 30 min at 20 °C, in a solution of 0.15 M or 0.3 M KCl, 10 mM $MgCl_2$, and 20 mM Tris–HCl (pH 7.5) with or without 1.5 mM ATP. Centrifugation was carried out at 60,000 rpm and 20 °C, in a solution of 0.15 M or 0.2 M KCl, 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5), 1 mM ATP, and 0.84 mg/ml native or treated myosin.

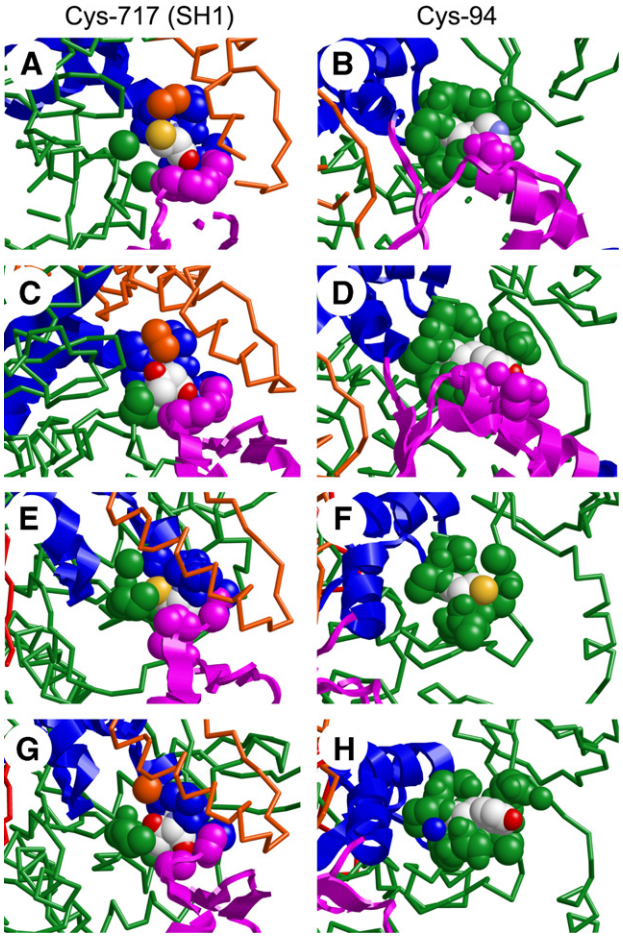

Fig. 4. Close-up view of the two reactive cysteines in myosin. Crystal structures: (A,B) chicken skeletal S1 with no nucleotide [41]; (C,D) *Dictyostelium* MD with no nucleotide [42]; (E,F) chicken gizzard MDE with MgADP·AlF_4^- [32]; and (G,H) *Dictyostelium* MD with MgADP·VO_4^- [43]. Cys-94 and Cys-717 of gizzard myosin and the corresponding residues of skeletal myosin or *Dictyostelium* myosin are shown as space-filling balls in white (carbon), blue (nitrogen), red (oxygen), and yellow (sulfur). Cys-94 is substituted with histidine in (B) and with phenylalanine in (D) and (H). Cys-717 is substituted with threonine in (C) and (G). All atoms located within 5.0 Å of these residues are also shown as space-filling balls. Except for Cys-94 and Cys-717, the same colors are used as in Fig. 1.

[2] The rotation of the converter we are now discussing is that coupled to nucleotide binding. Since the lever arm swing upon ATP binding has the direction opposite to that occurring in the power stroke, this swing is called the "reverse" or "recovery" stroke [25]. This movement of the lever arm becomes the restoring action necessary for the start of the next power stroke. Thus, the state after the rotation of the converter is called the pre-power stroke state.

and *Dictyostelium* MD with MgADP·VO_4^- are analogs for a state after the converter rotation. Before the rotation of the converter, Cys-717 (panels A and C) was exposed, but Cys-94 (panels B and D) was blocked by a part of the converter (magenta balls), whereas after the rotation of the converter, Cys-717 (panels E and G) was blocked by a part of the catalytic domain (green balls), but Cys-94 (panels F and H) was exposed. These observations are consistent with our experimental results that ATP inhibits the incorporation of AEDANS into the 68-kDa fragment (Fig. 2A) and accelerates its incorporation into the 24-kDa fragment (Fig. 2B), because the former contains Cys-717 and the latter contains Cys-94.

In the presence of ATP and in 0.15 M or 0.2 M KCl, the modification of SH1 was strongly inhibited by the formation of 10S myosin (Fig. 2A). Since the Mg–ATPase activity of 10S myosin is reportedly over 20-fold lower than that of 6S myosin [26,27], the turnover rate of the ATPase cycle must be very slow in these low KCl concentrations. We thus interpret that the inhibitory effect of ATP on the SH1 modification is further strengthened by the transition from the 6S to 10S form, because the chance for an encounter of IAEDANS with the nucleotide-free state of myosin in a fixed period of modification is decreased.

SH1-modified smooth muscle myosin cannot assume the 10S form because of incomplete rotation of the converter in the pre-power stroke state of the myosin

Panels A and C of Fig. 4 show that SH1 opens at the bottom of a cleft surrounded by parts of the catalytic domain (green strand), the relay helix (orange strand), and the converter (magenta ribbon). To determine whether this cleft in the nucleotide-free state is large enough to support the reaction of SH1 with IAEDANS, we investigated a model complex of AEDANS-fused myosin, using the crystal structure of skeletal S1 with no nucleotide. The atomic coordinates of S1 were fixed, and the structure of AEDANS-cysteine was annealed independently 20 times from 1000 K to 297 K. Each annealing generated a somewhat different structure of AEDANS-cysteine (an example is shown in panel A of Fig. 5), but every annealed structure was accommodated well in the deep cleft. From this result, we conclude that the cleft in the state before the rotation of the converter is large enough to allow the reaction with IAEDANS at its bottom. On the other hand, in the cleft of myosin in the pre-power stroke state, SH1 was blocked by both a loop projecting from the catalytic domain and a part of the converter as shown in panels E and G of Fig. 4. For example, the green balls represent Gly-118 and Leu-119, and the magenta balls depict Pro-722 and Thr-778 in these panels. To deduce how the SH1 modification affects the myosin structure in the pre-power stroke state, we tried to construct a putative structure of AEDANS-fused myosin, using the crystal structure of gizzard MDE with MgADP·AlF_4^-. After several trials, we found that AEDANS can pass through the narrow slit between two

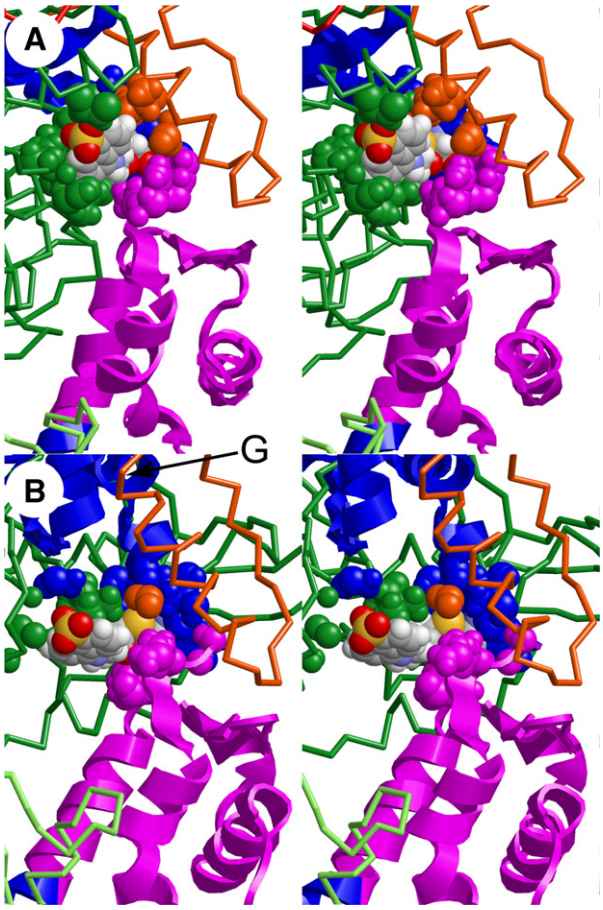

Fig. 5. Stereoview of AEDANS-modified myosins before (A) and after (B) rotation of its converter, simulated from the crystal structures of chicken skeletal S1 with no nucleotide [41] and chicken gizzard MDE with MgADP·AlF_4^- [32], respectively. AEDANS-cysteines are shown as space-filling balls in white (carbon), blue (nitrogen), red (oxygen), and yellow (sulfur). All atoms located within 5.0 Å of AEDANS-cysteines are also shown as space-filling balls. G in (B) indicates the position of Gly-709.

projections from the catalytic domain and the converter, when the C-terminal domain of MDE, starting from Gly-709, is rotated by ~15° around Gly-709 toward its position in the nucleotide-free state (panel B of Fig. 5). From this result, we conclude that the converter of the SH1-modified myosin cannot completely rotate toward its position in the pre-power stroke state of untreated myosin.

The incomplete rotation of the converter can explain why the SH1 modification inhibits the formation of 10S myosin and results in losses of the phosphorylation-dependent regulation of the basic and actin-activated Mg–ATPase activities of myosin. Wendt et al. [28,29] reconstructed a 3-D image of two inhibited heads of unphosphorylated gizzard HMM, by forming two-dimensional HMM crystalline arrays on a lipid monolayer and studying its structure by electron microscopy. In this image, the two heads were highly asymmetric, and the top of one head, which is referred as "blocked", interacts with parts of the catalytic (red and green balls in Fig. 6) and converter (magenta balls) domains, and the ELC (light green balls) of the second head named "free". Essentially the same image as that

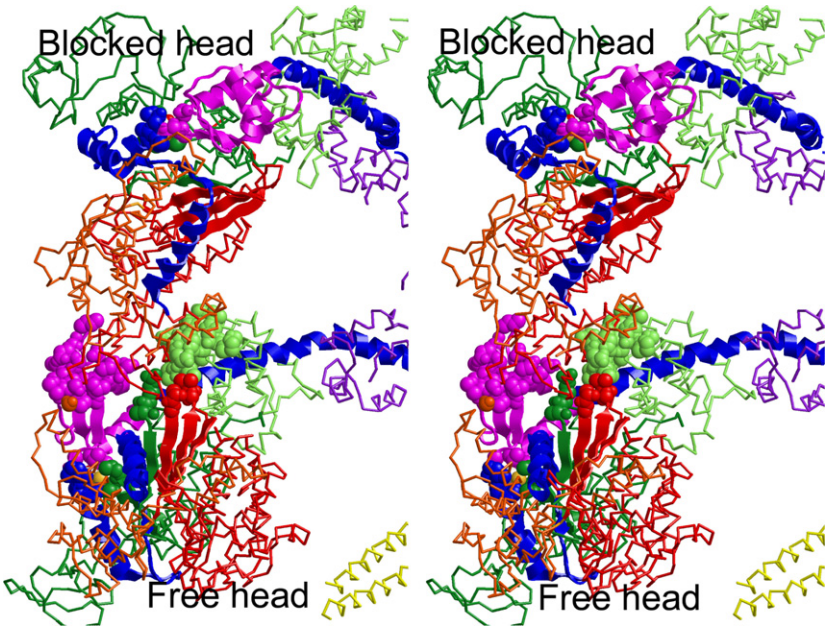

Fig. 6. Stereoview of the head-head interaction in 10S myosin. The three-dimensional model of the 10S conformation of smooth muscle myosin is adapted from Ref. [29]. In the blocked and free heads, the same colors are used as in Fig. 1. RLC and S2 are colored violet and yellow, respectively, but most of the rod is not shown. Seven-stranded β-sheets are indicated by ribbons colored green (strand 1, 113–118; strand 2, 120–125; strand 4, 170–175), dark blue (strand 3, 678–684), and red (strand 5, 459–467; strand 6, 249–256; and strand 7, 263–271). Cys-717 and atoms located within 5.0 Å of this cysteine are shown as space-filling balls. All free head atoms that are located within 5.0 Å of the upper 50-kDa subdomain (strand in red) of the blocked head are also shown as space-filling balls.

of unphosphorylated gizzard HMM was reported for the two heads of gizzard 10S myosin by Liu et al. [30] and Burgess et al. [31]. Since the 3-D images of both heads superimposed well on the crystal structure of gizzard MDE with MgADP·AlF_4^- [32], we can assume that the complete rotation of the converter seen in the crystal structure may be a prerequisite for the head–head interaction. If this assumption is true, then we can suggest that after the reaction of SH1 with IAEDANS, the top of the blocked head may no longer be accessible to the converter and ELC parts of the free head even in the pre-power stroke state because of the restriction of the bend (curved arrow in Fig. 1) of the blocked head around the converter region, and therefore 10S myosin is prevented from forming.

Regarding the inhibition of the actin-activated Mg–ATPase activity by the dephosphorylation, Wendt et al. [29] proposed quite different mechanisms for two heads in the inhibited state of smooth muscle HMM. According to them, blocking the actin-binding surface of the blocked head inhibits the interaction with actin, while the activity of the free head, which can bind actin, is inhibited through the stabilization of converter movements. The phosphorylation activates the actin-activated Mg–ATPase activity through a loss of the inhibitory action by the head–head interaction. Our results can be explained along the same line, i.e., the activation of the actin-activated Mg–ATPase activity of the unphosphorylated myosin by the SH1 modification is likely due to the modification-induced inhibition of the head–head interaction. In addition, we propose a mechanism for the inhibition of the basic Mg–ATPase activity by the 10S formation for the unphosphorylated (unmodified native) myosin. The mechanism is actually common to the above-mentioned mechanism for the inhibition of the actin-activated Mg–ATPase activity for the unphosphorylated myosin. Namely, the converter of the blocked head is stabilized by interacting at its top with the free head, while that of the free head is stabilized by the broad associations of the blocked head with parts of the free head's catalytic and converter domains and its ELC. The head–head interaction in the 10S myosin must be further stabilized by holding the blocked head firmly with the tail [31], thus its basic Mg–ATPase activity is inhibited by over 20-fold [26,27], because the rotation of the converter is an obligatory motion of the ATPase cycle. In 0.07 M KCl where we measured the basic and actin-activated Mg–ATPase activities of the modified myosins (Fig. 3A and B, respectively), myosin filaments are resistant to the dissociating action of ATP regardless of phosphorylation, and thus do not assume the 10S form [21]. However, we believe that the unphosphorylated (unmodified native) myosin in the filamentous form can form the essentially same head–head interaction in the presence of ATP as that observed with unphosphorylated gizzard HMM [28,29] or 10S myosin [30,31], although the head–head interaction of the filamentous form myosin is less stable than that of the 10S myosin. Both phosphorylation and modification inhibit the head–head interaction and therefore cause the activation of the basic Mg–ATPase activity, as well as the actin-activated Mg–ATPase activity of the unphosphorylated myosin.

The closure of the gate for the P_i release from the active site and the transition from the weak-binding state of activated myosin heads to the strong-binding state are also affected by incomplete rotation of the converter

As discussed above, the SH1 modification mimics the role of phosphorylation, in both the presence and absence of actin. However, it should be noted that effects of the modification and the phosphorylation are not identical, because the modification restricts the complete rotation of the converter regardless of the phosphorylation. Namely, the SH1 modification increased the basic Mg–ATPase activities of the unphosphorylated and phosphorylated myosins to the same value of about 0.06 s^{-1} $head^{-1}$ (Fig. 3A). We interpreted that there are the increase by a loss of the response to phosphorylation and the further threefold increase of the activity, which is independent of the phosphorylation. Because SH1 is connected with the "switch 2" loop of the active site via the so-called "relay helix" [32], it is natural to assume that the incomplete converter rotation of the SH1-modified myosin results in an incomplete closure of the gate ("back door") for the P_i release [33], leading to the threefold increase of the basic Mg–ATPase activity. The activation by the inhibition of the head–head interaction and the increase by incomplete closure of the gate for the P_i release are additive for the activity of the unphosphorylated modified myosin, while only the latter contributes for the activity of the phosphorylated modified myosin.

Additionally, the incomplete converter rotation by the SH1 modification has an inhibitory effect on myosin actively interacting with actin. The net actin activation of the phosphorylated myosin Mg–ATPase was reduced by over 90% (Fig. 3B) or about 60% [9] by modifying SH1 with IAEDANS or NEM, respectively. When myosin bearing ADP-P_i encounters actin, they initially form the weak-binding complex, which is followed by the transition from the weak- to the strong-binding state [34]. This weak-to-strong transition requires major conformational changes that result in the P_i release and the power stroke, and in phosphorylated native myosin, it is greatly accelerated by actin. The reduction of the net actin activation by the modification suggests that the incomplete converter rotation also affects the rate of the transition from the weak- to strong-binding state. Interestingly, the activity of skeletal myosin Mg–ATPase activated by regulated actin in the presence of Ca^{2+} was also reduced by 30–70% depending on the thiol reagent used for modifying SH1 [7]. Although the mechanism is still unclear, skeletal and phosphorylated smooth muscle myosins may have a common mechanism for these reductions.

The regulation of skeletal muscle contraction by troponin–tropomyosin may also be disabled by incomplete rotation of the converter

A study using a series of thiol reagents demonstrated that all of the thiol reagents tested abolished the Ca^{2+}-sensitivity of skeletal myosin Mg–ATPase activated by regulated actin [7]. This loss of Ca^{2+}-sensitivity was mainly due to an increase of the activity by 9- to 13-fold in the absence of Ca^{2+}, compared with a decrease by 1.4- to 3.3-fold in its presence. Structural similarities between skeletal and smooth muscle myosins suggest that Ca^{2+}-regulation of the skeletal contractile system may be also disabled by incomplete rotation of the converter of SH1-modified myosin. However, the mechanism for functional losses by the modification of skeletal SH1 must be different from that explained in the foregoing for smooth muscle myosin. Troponin-tropomyosin is an actin-binding regulatory system, and it can effectively inhibit the transition from the weak- to the strong-binding complex in the absence of Ca^{2+} by blocking part of the myosin-binding site on actin. However, tropomyosin does not strongly inhibit the actin-myosin interaction in the weak-binding complex initially formed upon actin binding, because studies of skeletal S1 Mg–ATPase activity with regulated actin indicated that Ca^{2+} accelerates the P_i release, but does not make big changes in the affinity of S1 for actin [35–38]. Therefore, the actin-myosin interfaces dominantly involved in the formation of the weak-binding complex must be different from those blocked by tropomyosin. The latter is mainly engaged in the transition from the weak- to strong-binding state. By using the atomic model of acto-S1 reported by Rayment et al. [39], Lorenz et al. suggested that on actin filaments, tropomyosin has overlaps with the cardiomyopathy loop or some neighboring loops of the upper 50-kDa subdomain of bound S1 [40]. Based on these results, we propose a mechanism that the SH1-modified myosin may disable the blocking of the actin-cardiomyopathy loop interaction by tropomyosin because of its abnormal conformation in the pre-power stroke state and thus no longer inhibit the activation of myosin Mg–ATPase by regulated actin even in the absence of Ca^{2+}. However, further studies are needed to determine whether this interpretation is reasonable.

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